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(54) Title: HUMAN INTERLEUKIN-4 MUTEINS

Sequence of GluAlaGluAla-hIL-4(Asp62, Asp129)

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GTA

←---α-factor-------→|←------------hIL-4 mutein--------→
CCT TTA GAT AAA AGA GAA GCT GAA GCT CAC AAG TGC CAT ATC ACC   90
Pro Leu Asp Lys Arg Glu Ala Glu Ala His Lys Cys Asp Ile Thr   30

TTA CAG GAG ATC ATC AAA ACT TTG AAC AGC CTC ACA GAG CAG AAG  135
Leu Gln Glu Ile Ile Lys Thr Leu Asn Ser Leu Thr Glu Gln Lys   45

                          ↓BstEII                      NheI
ACT CTG TGC ACC GAG TTG ACG GTA ACC GAC ATC TTT GCT GCT AGC  180
Thr Leu Cys Thr Glu Leu Thr Val Thr Asp Ile Phe Ala Ala Ser   60

                                    ↓PstI
AAG GAC ACA ACT GAG AAG GAA ACC TTC TGC AGG GCT GCG ACT GTG  225
Lys Asp Thr Thr Glu Lys Glu Thr Phe Cys Arg Ala Ala Thr Val   75

CTC CGG CAG TTC TAC AGC CAC CAT GAG AAG GAC ACT CGC TGC CTG  270
Leu Arg Gln Phe Tyr Ser His His Glu Lys Asp Thr Arg Cys Leu   90

GGT GCG ACT GCA CAG CAG TTC CAC AGG CAC AAG CAG CTG ATC CGA  315
Gly Ala Thr Ala Gln Gln Phe His Arg His Lys Gln Leu Ile Arg  105

TTC CTG AAA CGG CTC GAC AGG AAC CTC TGG GGC CTG GCG GGC TTG  360
Phe Leu Lys Arg Leu Asp Arg Asn Leu Trp Gly Leu Ala Gly Leu  120

↓EcoRI                                  ↓SalI
AAT TCC TGT CCT GTG AAG GAA GCC CAC CAG TCG ACG TTG GAA AAC  405
Asn Ser Cys Pro Val Lys Glu Ala Asp Gln Ser Thr Leu Glu Asn  135

TTC TTG GAA AGG CTA AAG ACG ATC ATG AGA GAG AAA TAT TCA AAG  450
Phe Leu Glu Arg Leu Lys Thr Ile Met Arg Glu Lys Tyr Ser Lys  150

TGT TCG AGC TGA                                             495
Cys Ser Ser End                                             153
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(57) Abstract

Recombinant biologically active human IL-4 (rhIL-4) mutant analog proteins in which N-linked glycosylation sites have been inactivated.

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TITLE

Human Interleukin-4 Muteins

BACKGROUND OF THE INVENTION

The present invention relates generally to lymphokines, and particularly to recombinant interleukin-4 muteins or analog proteins, which induce clonal expansion and maturation of activated B cells and augment generation of cytotoxic T cells.

B lymphocytes, or B cells, are the precursors of antibody-secreting plasma cells. B cells derive from hematopoietic stem cells located in the bone marrow, via an intermediary cell class known as pre-B cells. B cells are distinguished from pre-B cells by the expression of surface-bound immunoglobulin capable of binding specific antigens. B cells are activated by binding of antigen to membrane receptors, provided that the B cells also interact with specific helper T cells or bind certain soluble growth and differentiation factors. B cell activation is a sequential process involving proliferation and differentiation phases. In the proliferation phase, activated B cell clones multiply to provide an expanded number of cells capable of reacting with the activating antigen. In the differentiation phase, a portion of the activated B cells mature and secrete immunoglobulin as circulating plasma cells. Separate T lymphocyte-derived cytokines, which were first designated "B cell growth factor" (BCGF) and "B cell differentiation factor" (BCDF), are involved in the regulation of proliferation and differentiation phases. Alternative terms for BCGF include "B cell stimulating factor 1" (BSF-1), and "interleukin-4" (IL-4), the latter now being preferred.

Howard et al., J. Exp. Med. 155:914 (1982), and Farrar et al., J. Immunol. 131:1838 (1983) described a B cell stimulating factor derived from mitogen-stimulated murine T cells which stimulated B cell proliferation. Following this disclosure, a number of laboratories reported similar murine activities in media conditioned by T cell hybridomas, cloned T cells, and normal T cells. See, e.g., Roehm et al., J. Exp. Med. 160:679 (1984); Noelle et al., Proc. Natl. Acad.

Sci. USA 81:6149 (1984); Oliver et al., Proc. Natl. Acad. Sci. USA 82:2465 (1985); Rabin et al., Proc. Natl. Acad. Sci. USA 82:2935 (1985); and Vitetta et al., J. Exp. Med. 162:1726 (1985). Purification to homogeneity of a murine BSF-1/IL-4 species was reported by Grabstein et al., J. Exp. Med. 163:1405 (1986).

Isolation of cDNAs encoding proteins having murine BSF-1/IL-4 activity was recently reported by Noma et al., Nature 319:640 (1986) and Lee et al., Proc. Natl. Acad. Sci. USA 83:2061 (1986). Yokota et al., Proc. Natl. Acad. Sci. USA 83:5894 (1986) isolated a human cDNA clone having homology to mouse IL-4. The human cDNA encoded a protein of 153 amino acid residues including a possible signal peptide. Supernatants of monkey COS-1 cells transfected with this cDNA were capable of inducing proliferation of anti-IgM-exposed human B cells. This activity is analogous to a known property of murine IL-4 in conjunction with murine B cells.

IL-4 also stimulates growth and differentiation of factor-dependent T cell and myeloid cell classes. Grabstein et al., supra, reported that murine IL-4 induced proliferation of murine IL-2-dependent and IL-3-dependent T cell lines. Other studies have indicated that IL-4 stimulates mast cell proliferation and macrophage differentiation.

The availability of significant quantities of purified IL-4 has facilitated studies of B cell ontogeny and function, and illuminated potential therapeutic uses for this lymphokine. Among the uses presently contemplated for recombinant human IL-4 are treatment of immune deficiency diseases characterized by B cell cytopenias, and induction of B cell differentiation as a treatment for certain B cell related lymphocytic leukemias. IL-4 might also be used to induce and maintain continuous cultures of immunoglobulin-secreting B cells to provide a source of human monoclonal antibodies. The present applicant have discovered that IL-4 induces proliferation and differentiation of cytolytic T cells previously exposed to a mitogenic stimulus; this observation indicates that IL-4 can be employed as a therapeutic lymphokine in treatment of viral infection and certain neoplastic conditions.

SUMMARY OF THE INVENTION

The present invention is directed to recombinant human IL-4 proteins produced using yeast expression systems. Preferred are analog proteins including those having inactivated asparagine-linked glycosylation sites, for example, hIL-4 (Asp^{62}, Asp^{129}). This invention also concerns DNA sequences encoding the muteins, recombinant expression vectors comprising the DNA sequences, and processes for making the muteins comprising culturing microorganisms transformed with the recombinant expression vectors. The present invention also provides a method for inducing proliferation of and lytic activity in a population of antitumor cytolytic T lymphocytes (CTL), comprising contacting T cells with a composition comprising a biologically effective quantity of IL-4 in combination with a physiologically acceptable carrier or diluent. In a related aspect, the present invention provides methods for inducing proliferation and activation of antitumor or antiviral cytolytic T lymphocytes in a mammal, e.g., a human, comprising administering a therapeutically effective quantity of a human IL-4 therapeutic composition.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 depicts the nucleotide sequence and corresponding amino acid sequence of wild-type native human IL-4.

FIGURE 2 depicts the nucleotide sequence of a DNA sequence encoding the hIL-4 mutein GluAlaGluAla-hIL-4(Asp^{62}, Asp^{129}).

FIGURES 3-5 schematically illustrate the construction of a yeast expression vector for production of the hIL-4 mutein GluAlaGluAla-hIL-4(Asp^{62}, Asp^{129}).

FIGURE 6 is a plot showing augmentation of cytolytic T cell generation in primary mixed leukocyte cultures (MLC) by IL-4 and IL-2.

FIGURE 7 is a plot illustrating induction of cytolytic activity in long-term MLC by IL-4 and IL-2.

DETAILED DESCRIPTION OF THE INVENTION

As detailed herein, a cDNA comprising a nucleotide sequence encoding native human IL-4 was isolated from a cDNA library prepared by reverse transcription of polyadenylated RNA isolated from human

peripheral blood T lymphocytes. Synthetic oligonucleotide probes having sequence homology to N-terminal and C-terminal regions of the native human sequence were employed to screen the library by conventional DNA hybridization techniques. Clones from the library comprising plasmid DNAs which hybridized to the probes were isolated and analyzed by restriction endonuclease cleavage, agarose gel electrophoresis, and additional hybridization experiments ("Southern blots") involving the electrophoresed fragments. After isolating a single clone which hybridized to both the N-terminal and C-terminal oligonucleotide probes, the hybridizing segment was cleaved to provide a smaller restriction fragment bearing the hIL-4 gene, which was then subcloned and sequenced by conventional techniques. The cDNA encoding mature hIL-4 was then digested with selected restriction endonucleases and reassembled using synthetic oligonucleotides providing predetermined codon changes. The resulting mutant cDNA sequence was inserted into a yeast expression vector under control of a particular promoter. The vector was used to transform an appropriate yeast expression strain, which was grown in culture under conditions promoting derepression of the yeast promoter. The resulting yeast-conditioned culture supernatant provided a protein having hIL-4 biological activity, which was purified as described below.

Definitions

"Human interleukin-4" and "hIL-4" refer to a human endogenous secretory protein capable of inducing maturation and proliferation of human B cells, which comprises an amino acid sequence which is substantially homologous to all or a significant part of the sequence set forth in FIGURE 1. Other designations for this molecule include "B-cell stimulating factor" and "B-cell growth factor".

"DNA sequence" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, which has been derived from DNA isolated at least once in substantially pure form, i.e., in a quantity or concentration enabling identification, manipulation, and recovery of the segment and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. "Nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides. "Recombinant expression vector" refers to a

plasmid comprising a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, and (2) a structural or coding sequence which is transcribed into mRNA and translated into protein. Preferably, the transcriptional unit includes a leader sequence enabling extracellular secretion of translated protein by a host cell. "Recombinant expression system" means a combination of an expression vector and a suitable host microorganism. Yeast expression systems, particularly those employing Saccharomyces cerevisiae, are preferred.

"Mutant amino acid sequence" refers to a polypeptide encoded by a nucleotide sequence intentionally made variant from a native sequence. "Mutant protein" or "mutein" means a protein comprising a mutant amino acid sequence. "Substantially homologous," which can refer both to nucleic acid and amino acid sequences, means that a particular subject sequence, for example, a mutant sequence, varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which do not result in an adverse functional dissimilarity between reference and subject sequences. For purposes of the present invention, sequences having greater than 80 percent homology and equivalent biological specific activity are to be considered substantially homologous sequences within the scope of the present invention. Sequences having lesser degrees of homology and comparable bioactivity are to be considered equivalents. "Native sequence" refers to an amino acid or nucleic acid sequence which is identical to a wild-type or native form of a gene or protein. "N-glycosylation site" is defined below. The term "inactivate", as used in defining the present invention, means to alter a selected N-glycosylation site to eliminate amino acid residues enabling covalent bonding of oligosaccharide moieties.

Assays for Human IL-4 Activity

Human IL-4 activity can be observed in cultures of human B cells derived, for example, from suspensions of human tonsillar cells. Enriched B cell populations can be prepared by rosetting T-cells with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes followed by Ficoll-Histopaque (Sigma Chemical Corp., St. Louis, MO, USA) to

eliminate T cells, and Sephadex G10 filtration to remove monocytes, granulocytes, and activated B cells. Following enrichment, B cell preparations can be frozen in liquid N_2 prior to use.

For assay, frozen B cells are thawed, washed, and cultured at 10^5 cells per well in 100 µl of RPMI 1640 medium containing 10% fetal calf serum, $5x10^{-5}$M 2-mercaptoethanol, appropriate dilutions of the sample to be tested, and 12.5 µg/ml of $F(ab')_2$ fragments goat anti human IgM purified by affinity chromatography. Cultures are incubated for 68-72 hours. During the final 16 hours of the incubation period, the cells receive 0.5 µCi [^{3}H]-thymidine at a specific activity of 75 Ci/mmole. Cultures are then harvested onto glass fiber filters and incorporation of radiolabel determined by scintillation counting.

Details regarding analogous assays for murine IL-4 activity are to be found in the references reviewed by Brooks et al., Methods Enzym. 116:372 (1985).

In assays for hIL-4 activity, units of activity are calculated by reference to the quantity of hIL-4 which induces 50% of maximal thymidine incorporation. For example, if a 100 µl sample generates one-half maximal thymidine incorporation at a dilution of 1:20, one unit is defined as the activity contained in 1/20 of 100 µl, or 5 µl. The sample would therefore contain 1000 divided by 5, or 200 units per milliliter (U/ml) of hIL-4 activity.

Nucleotide and Amino Acid Sequences of hIL-4 Proteins

The nucleotide and deduced amino acid sequences of a cDNA sequence encoding a wild-type hIL-4 protein are set forth in FIGURE 1. In FIGURE 1, nucleotides are numbered beginning with the ATG codon corresponding to the N-terminal methionine of the full-length native polypeptide. Similarly, amino acids are numbered from this methionine residue. The native protein includes a leader sequence of 23 or 25 amino acids preceding a histidine residue providing the N-terminus of the mature secreted protein. On the basis of comparison to the murine homologue of hIL-4, His^{23} is the predicted N-terminus. However, parallel expression experiments have indicated equivalent biological activity for proteins having His^{23} or His^{25} as the N-terminal amino acid residue.

FIGURE 2 indicates the nucleotide and encoded amino acid sequence of a synthetic gene encoding a hIL-4 mutein, GluAlaGluAla-hIL-4-(Asp^{62}, Asp^{129}), which represents a preferred embodiment within the scope of the present invention.

Construction of Analog Sequences and Muteins

Numerous DNA constructions including all or part of the nucleotide sequence of FIGURE 1, in conjunction with oligonucleotide cassettes comprising additional useful restriction sites, can be prepared as a matter of convenience. This invention concerns certain analog proteins or muteins which are substantially homologous to the native sequence of hIL-4, yet contain one or more intentional amino acid substitutions, deletions, or insertions not adversely affecting activity.

Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes a mutein having the desired amino acid insertion, substitution, or deletion. This approach is illustrated by FIGURES 3-6.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Bauer et al., Gene 37:73 (1985); Craik, Biotechniques, January 1985, 12-19; Smith et al., Genetic Engineering: Principles and Methods (Plenum Press, 1981); and U. S. Patent 4,518,584 disclose suitable techniques, and are incorporated by reference herein.

For either approach, conventional techniques for oligonucleotide synthesis are suitable, for example, the triester synthesis procedures disclosed by Sood et al., Nucl. Acid Res. 4:2557 (1977) and Hirose et al., Tet. Lett. 28:2449 (1978).

In site-specific mutagenesis, a strand of the gene to be altered is cloned into an M13 single-stranded phage or other appropriate vector to provide single-stranded DNA comprising either the sense or antisense strand corresponding to the gene to be altered.

This DNA is annealed to a fragment of M13 phage to provide a gapped duplex, which is then hybridized to an oligonucleotide primer. The primer is complementary to the sequence surrounding the codon to be altered, but comprises a codon (or an antisense codon complementary to such codon) specifying the new amino acid at the point where substitution is to be effected. If a deletion is desired, the primer will lack the particular codon specifying the amino acid to be deleted, while maintaining the correct reading frame. If an insertion is desired, the primer will include a new codon, at the appropriate location in the sequence, specifying the amino acid to be inserted. Preferably, the substitute codon, deleted codon, or inserted codon is located at or near the center of the oligonucleotide.

The size of the oligonucleotide primer employed is determined by the need to optimize stable, unique hybridization at the mutation site with the 5′ and 3′ extensions being of sufficient length to avoid editing of the mutation by the exonuclease activity of the DNA polymerase employed to fill gaps. Thus, oligonucleotides used in accordance with the present invention will usually contain from about 15 to about 25 bases. Oligonucleotides of greater size are not needed.

The oligonucleotide primer is then hybridized to the gapped duplex having a single-stranded template segment containing the gene to be altered. The primer is then extended along the template strand by reaction with DNA polymerase I (Klenow fragment), T4 DNA polymerase, or other suitable DNA polymerase. The resulting double stranded DNA is then converted to closed circular DNA by treatment with a DNA ligase, for example, T4 DNA ligase, and the resulting heteroduplex employed to transfect a suitable host strain, for example E. coli JM105 (Bethesda Research Laboratories, Gaithersburg, MD, USA). Replication of the heteroduplex by the host provides progeny of both strands. The transfected cells are then plated to provide plaques, which are screened using a labelled oligonucleotide corresponding to that used in the mutagenesis procedure. Conditions are employed which result in preferential hybridization of the primer to the mutated DNA but not to the progeny of the parent strand. DNA containing the mutated gene is then isolated and spliced into a suitable expression

vector, and the vector employed to transform a host strain. The host strain is then grown in culture to provide the analog protein.

The particular mutation strategy forming the basis of the present invention is described below.

Inactivation of N-glycosylation Sites

Many secreted proteins acquire covalently attached carbohydrate units following translation, frequently in the form of oligosaccharide units linked to asparagine side chains by N-glycosidic bonds. Both the structure and number of oligosaccharide units attached to a particular secreted protein can be highly variable, resulting in a wide range of apparent molecular masses attributable to a single glycoprotein. Human IL-4 is a secreted glycoprotein of this type. Attempts to express glycoproteins in recombinant systems can be complicated by the heterogeneity attributable to this variable carbohydrate component. For example, purified mixtures of recombinant glycoproteins such as human or murine granulocyte-macrophage colony stimulating factor (GM-CSF) can consist of from 0 to 50% carbohydrate by weight. Miyajima et al., EMBO Journal 5:1193 (1986) reported expression of a recombinant murine GM-CSF in which N-glycosylation sites had been mutated to preclude glycosylation and reduce heterogeneity of the yeast-expressed product.

The presence of variable quantities of associated carbohydrate in recombinant secreted glycoproteins complicates purification procedures, thereby reducing yield. In addition, should the glycoprotein be employed as a therapeutic agent, a possibility exists that recipients will develop allergic reactions to the yeast carbohydrate moieties, requiring therapy to be discontinued. For these reasons, biologically active, homogeneous analogs of immunoregulatory glycoproteins having reduced carbohydrate are desirable for therapeutic use.

Functional mutant analogs of human IL-4 having inactivated N-glycosylation sites can be produced by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques as described above. These analog proteins can be produced in a homogeneous, reduced-carbohydrate form in good yield using yeast expression systems. The present invention concerns analog forms of human IL-4

comprising at least one amino acid substitution, deletion, or insertion inactivating an N-glycosylation site.

N-glycosylation sites in eukaryotic proteins are characterized by the amino acid triplet Asn-A^1-Z, where A^1 is any amino acid, and Z is Ser or Thr. In this sequence, asparagine (Asn) provides a side chain amino group for covalent attachment of carbohydrate. Such a site can be eliminated by substituting another amino acid for Asn or for residue Z, deleting Asn or Z, or inserting a non-Z amino acid between A^1 and Z, or an amino acid other than Asn between Asn and A^1. Preferably, substitutions are made conservatively; i.e., the most preferred substitute amino acids are those having physicochemical characteristics resembling those of the residue to be replaced. Similarly, when a deletion or insertion strategy is adopted, the potential effect of the deletion or insertion upon biological activity should be considered.

Thus, an analog hIL-4 according to the present invention is a protein having a mutant amino acid sequence which is substantially homologous to the native sequence of hIL-4, wherein at least one occurrence Asn-A^1-Z in the native sequence has been replaced in the mutant sequence by Asn-A^2-Y or X-A^2-A^3, where

A^1, A^2, and A^3 are the same or different and can be any amino acid,

X is any amino acid not Asn;

Y is any amino acid not Z; and

Z is Ser or Thr.

Preferably, all occurrences of Asn-A^1-Z in the native sequence are replaced in the mutant sequence by Asn-A^2-Y or X-A^2-A^3.

Referring now to the sequence of hIL-4 set forth in FIGURE 1, it can be seen that the native protein contains two putative N-glycosylation sites, the first being the triplet AsnThrThr beginning at residue 62, and the second being AsnGlnSer beginning at residue 129. Appropriately conservative substitute amino acids for Asn include Asp, Gln, Glu, Ala, Gly, Ser, and Thr, of which Asp, Gln, and Glu are preferred. Where Z is Ser, appropriate substitutes are Met, Leu, Ile, Val, Asp, Gln, Glu, or Asn; of which Met, Leu, Ile, and Val are preferred. Where Z is Thr, conservative substitutions are Val,

Glu, Asp, Gln, Gly, or Ala, preferably Val, Glu, Asp or Gln. In the context of the present invention, preferred substitutions to inactivate the hIL-4 N-glycosylation sites are substitution of Val for Thr^{64} or Asp for Asn^{62}, and Asp for Asn^{129}.

Other conservative amino acid substitutions could be made to provide protein lacking N-glycosylation sites. Muteins bearing such substitution are considered to be equivalents of those specifically disclosed and claimed herein. Ranking substitute amino acids by order of preference for substitution at these positions provides the following Table 1:

Table 1: hIL-4 Amino Acid Substitutions

Position:	62	64	129	131
Wild type:	Asn	Thr	Asn	Ser
Most preferred:	Asp	Val	Asp	Met
Second Order of preference:	Glu	Glu	Glu	Leu
	Gln	Gln	Gln	Ile
		Asp		Val
Third Order of preference:	Ala	Gly	Ala	Asp
	Gly	Ala	Gly	Gln
	Ser		Ser	Glu
	Thr		Thr	Asn

Deletion or Substitution of Cysteine Residues

The present invention also contemplates muteins of hIL-4 in which cysteine residues not essential to biological activity have been deleted or replaced with other amino acids to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bond formation. The native sequence of hIL-4 comprises six cysteine residues, at positions 27, 48, 70, 89, 122, and 151 (see FIG. 1). The first five cysteines have counterparts in the murine homologue, while the last cysteine does not. Thus, the last residue is an appropriate candidate for substitution or deletion.

Site specific mutagenesis or oligonucleotide substitution procedures can be employed to delete particular cysteine residues, or provide conservative substitutions. Preferred amino acids for substitution are neutral amino acids such as Gly, Ala, Val, Leu, Ile,

Tyr, Phe, His Trp, Ser, Thr, or Met. Of the foregoing, Ser and Thr are preferred.

Inactivation of KEX2 Protease Recognition Sites

Appropriate mutagenesis procedures can also be employed to inactivate KEX2 protease processing sites by deletion, addition, or substitution of residues to alter Arg-Arg, Arg-Lys, or Lys-Arg pairs in a manner eliminating the occurrence of adjacent basic residues. It should be noted that Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative approach to inactivating KEX2 sites. The resulting muteins are less susceptible to cleavage by the KEX2 protease at locations other than the α-factor leader sequence where cleavage upon secretion is intended.

Referring to FIG. 1, a Lys-Arg pairing occurs at position 123 of the hIL-4 native sequence. Substitution of a non-Arg amino acid for Lys^{123} or Arg^{124} provides a mutant hIL-4 free of internal Arg-Arg, Lys-Arg, or Arg-Lys KEX2 processing sites. Comparison with the mouse IL-4 sequence suggests that deletion of Lys^{123} is a conservative mutagenesis strategy, and is therefore preferred. Alternatively, Lys can be substituted for Arg^{124}.

Modification of Yeast KEX2 Protease Recognition Sites

A preferred expression system for the IL-4 proteins of this invention employs the yeast α-factor leader sequence to induce secretion of recombinant protein by a yeast host. Ideally, this system is configured such that the yeast KEX2 protease cleaves the α-factor leader from the N-terminus of the desired protein upon secretion. An α-factor leader-hIL-4 protein construction having a Lys-Arg KEX2 protease site immediately adjacent to the N-terminal His residue of wild-type was not always cleaved upon secretion by recombinant yeast. When the tetrapeptide sequence Glu-Ala-Glu-Ala was inserted between the Lys-Arg KEX2 recognition site and the N-terminus of hIL-4, more efficient cleavage at the KEX2 site was achieved. The resulting product is an hIL-4 protein having the tetrapeptide Glu-Ala-Glu-Ala at the N-terminus. Potentially, these

residues could be removed in vivo in a yeast strain capable of over-expressing the yeast STE13 gene product, dipeptidyl aminopeptidase A, which cleaves N-terminal Glu-Ala pairs. However, the presence of the Glu-Ala-Glu-Ala sequence at the N-terminus has not been observed to provide any significant difference in the biological activity of the analog relative to the wild-type protein.

Protein Expression in Recombinant Yeast Systems

As noted previously, yeast is preferred for expression of analog and native forms of recombinant human IL-4. An exemplary expression vector is pBC104 (ATCC 67,232) which contains DNA sequences from pBR322 for selection and replication in E. coli (Ap^r gene and origin of replication) and yeast DNA sequences including a glucose-repressible alcohol dehydrogenase 2 (ADH2) promoter. The ADH2 promoter has been described by Russell et al., J. Biol. Chem. 258:2674 (1982) and Beier et al., Nature 300:724 (1982). Plasmid pBC104 also comprises the Trp1 gene as a selectable marker and the 2μ origin of replication. Adjacent to the promoter is the α-factor leader sequence enabling secretion of heterologous proteins from a yeast host. The α-factor leader sequence is modified to contain, near its 3′ end, an Asp^{718} (KpnI) restriction site to facilitate fusion of this sequence to foreign genes. pBC104 also comprises a cDNA insert encoding wild-type hIL-4. Details regarding the construction of this plasmid are provided below.

Alternative expression vectors are yeast vectors which comprise an α-factor promoter, for example pYαfHuGM (ATCC 53157), which bears the wild-type human GM-CSF gene. Others are known to those skilled in the art. The construction of pYαHuGM is described in published European Patent Application EP-A-183,350.

Selection of appropriate yeast strains for transformation will be determined by the nature of the selectable markers and other features of the vector. Appropriate S. cerevisiae strains for transformation by pBC104 or pYαHuGM, and various constructions derived from those vectors, include strains X2181-1B, available from the Yeast Genetic Stock Center, Berkeley, CA, USA [see below], having the genotype α trp1 gal1 ade1 his2; J17 (ATCC 52683; α his2 ade1 trp1 met14 ura3); and IL166-5B (ATCC 46183; α his1 trp1). A particularly

preferred expression strain, XV2181, is a diploid formed by mating two haploid strains, X2181-1B, available from the Yeast Genetic Stock Center, Department of Biophysics and Medical Physics, University of California, Berkeley, CA 94702, USA; and XV617-1-3B, available from the Department of Genetics, University of Washington, Seattle, WA 98105, USA, or Immunex Corporation, 51 University Street, Seattle, WA 98101, USA. A suitable transformation protocol is that described by Hinnen, et al., Proc. Natl. Acad. Sci. USA 75:1929 (1978), selecting for Trp^+ transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 µg/ml adenine and 20 µg/ml uracil.

Host strains comprising pBC104 or other constructions comprising the ADH2 or α-factor promoters are grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 µg/ml adenine and 80 µg/ml uracil. Derepression of the ADH2 promoter occurs upon exhaustion of medium glucose.

Purification of rhIL-4 Proteins

Recombinant human IL-4 proteins resulting from fermentation of yeast strains can be purified by single or sequential reversed-phase HPLC steps on a preparative HPLC column, by methods analogous to those described by Urdal et al., J. Chromatog. 296:171 (1984), and Grabstein et al., J. Exp. Med. 163:1405 (1986).

For example, yeast-conditioned medium containing rhIL-4 can be filtered through a 0.45 µ filter and initially purified by batch adsorption and elution from a cation exchange matrix, for example, S-Sepharose. Pooled fractions from the batch adsorption/elution step can then be pumped, at a flow rate of 100 ml/min, onto a 5 cm x 30 cm column packed with 10-20 µ reversed phase silica (Vydac, The Separations Group, Hesperia, CA, USA). The column can be equilibrated in 0.1% trifluoroacetic acid in water prior to the application of the yeast-conditioned medium and then flushed with this solvent following application of the medium to the column until the optical absorbance at 280 nm of the effluent approaches baseline values. At this time, a gradient of 0.1% trifluoroacetic acid in acetonitrile can be established that leads from 0 to 60-100% Solvent B at a rate of change

of 1-2% per minute and at a flow rate of 100 ml/min. At a suitable time (10-20 minutes) following initiation of the gradient, one minute fractions are collected and aliquots of the fractions analyzed for protein content by polyacrylamide gel electrophoresis and fluorescamine protein determination. Additional HPLC or cation-exchange chromatographic steps can be employed if indicated.

Utility

HIL-4 proteins represent promising therapeutic agents for treatment of immune deficiencies and neoplastic conditions. In such therapy, a hIL-4 protein in the form of a purified composition comprising the protein in combination with a physiologically acceptable carrier or diluent is administered by continuous parenteral infusion, subcutaneous injection, or other suitable means at an dosage rate effective to induce proliferation of B-cells and/or T-cells. Suitable dosages for IL-4 therapy, as indicated by animal studies, are from 0.1 to 100 µg/kg body weight per day. Alternatively, the protein can be used in forms of adoptive immunotherapy wherein particular immune cell classes are isolated, expanded in vitro in the presence of a hIL-4 protein, and readministered with additional hIL-4 as means of inducing tumor regression. Optionally, hIL-4 proteins can be used in conjunction with human interleukin-2.

These approaches to cancer therapy are suggested by the observation that purified murine IL-4 enhances the generation of cytolytic T lymphocytes in primary mixed leukocyte cultures, and induces cytolytic activity in populations of mixed leukocytes previously exposed to antigen from allogeneic cells.

Cytolytic T lymphocytes (CTL), also known as cytotoxic or effector T cells, are receptor-bearing, antigen-specific lymphocytes. Alloreactive CTL lyse target cells that display major histocompatibility gene complex (MHC) antigens identical to those of the allogeneic cells used to stimulate or induce the cytolytic cells. CTL specific for viral and/or tumor antigens are "restricted" in their recognition of antigens, in that antigen-bearing target cells must also display MHC antigens identical to those of the CTL themselves. CTL control viral replication by killing cells expressing virus-associated membrane antigens, and have also been indirectly

implicated in immune surveillance and destruction of certain neoplastic cell types.

CTL generation is studied most simply in mixed leukocyte cultures (MLC), wherein lymphocytes from genetically dissimilar (allogeneic) individuals are cocultured to induce T cell proliferation. Such T cells are specific for foreign MHC antigens, (present on cells of one individual and not the other) and are referred to as alloreactive T cells. CTL activation and differentiation require participation by CTL precursor cells, T "helper" cells, and accessory cells of monocyte/macrophage lineage. CTL response is initiated upon antigen recognition by particular T cell populations; exposure to appropriate antigen triggers lymphokine receptor expression on CTL precursors and lymphokine secretion by helper T cells. Lymphokine binding by CTL precursors induces proliferation and presumably differentiation of antigen-activated CTL precursors to a cytolytic state. However, a CTL precursor need not necessarily proliferate in order to attain its cytolytic potential; the ability to kill is apparently a differentiated function.

The T cell mediated lytic cycle begins with cell-to-cell contact between a viable effector cell and a target cell bearing the appropriate determinant. Unlike natural killer (NK) cells, which direct cytolytic activity to a broad spectrum of target cells without an overt requirement for antigen activation, CTL lyse with discriminating specificity. Following contact and adhesion of effector and target, a so-called "lethal hit" is administered, in which membrane permeability of the target is disrupted. This event results in osmotic swelling and the ultimate loss of cytoplasm. The effector cell retains the ability to recognize and lyse additional target cells.

The growth and differentiation of CTL is regulated by soluble growth hormones, of which interleukin-2 (IL-2) is considered to be of prime importance. It has now been found that IL-4 also profoundly influences the generation of functionally active CTL. In particular, IL-4 acts as a potent helper factor for the generation of CTL in primary mixed leukocyte culture (MLC) and induces cytolytic activity in <u>in vitro</u> primed, MLC memory populations. Direct comparison of

purified recombinant IL-4 and IL-2 has revealed IL-4 to be more potent than IL-2 in augmenting CTL generation in primary MLC. The two lymphokines differ in that IL-2, but not IL-4, induces a lytic population in cultures of unprimed cells in the absence of an overt antigenic stimulus. The specificity of cytolysis induced by IL-4 may have important therapeutic ramifications; the efficacy of adoptive immunotherapy may be enhanced if side effects attributable to introduction of non-specific lymphokine-activated killer (LAK) cells (e.g., in IL-2 LAK therapy) are reduced.

In related observations, recombinant IL-4 has been shown to effectively induce proliferation of mitogen-activated T-cells, thymocytes, memory T cells, and alloreactive T-cell clones of different functional subtypes, including CTL. IL-4 has been found to be as effective a stimulus as IL-2 for inducing proliferation of mitogen-activated murine spleen cells bearing the Lyt2+ surface antigen. Thus, it is apparent that IL-4 is an important regulator of T cell growth and function.

The following discussion is intended to provide additional details regarding particular aspects of the present invention. In the experiments described below, standard molecular biological techniques were followed as described in Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, 1982) for the restriction enzyme digestion of DNA, purification of DNA fragments by gel electrophoresis, ligation of DNA fragments, transformation into E. coli (strain RR1 was used throughout), and analysis and verification of constructs by restriction enzyme digestion.

Example 1: Isolation of cDNA encoding Wild-Type hIL-4 and Expression of Active Protein Using a Yeast Expression System

Synthetic oligonucleotides were constructed complimentary to N and C terminal coding region sequences of human IL-4. The N-terminal probe had the sequence 5′-CAGTTGGGAGGTGAGACCCAT-3′, while the C-terminal probe had the sequence 5′-TCAGCTCGAACACTTTGAATA-3′. The method of synthesis was a standard automated triester method substantially similar to that disclosed by Sood et al., Nucleic Acids Res. 4:2557 (1977) and Hirose et al., Tet. Lett. 28:2449 (1978).

Following synthesis, the oligonucleotide was deblocked and purified by Sephadex G-50 chromatography followed by preparative gel electrophoresis. The oligonucleotides were terminally radiolabelled with ^{32}P using ^{32}P-ATP and T4 polynucleotide kinase by standard techniques, such as those disclosed by Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory 1982), for use as screening probes.

A cDNA library was constructed by reverse transcription of polyadenylated mRNA isolated from total mRNA extracted from human peripheral blood T lymphocytes (PBT) stimulated with phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA). The cDNA was rendered double-stranded using DNA polymerase I and T4 DNA polymerase, methylated with EcoRI methylase to protect EcoRI cleavage sites within the cDNA from subsequent cleavage with EcoRI, ligated to EcoRI linkers, digested with EcoRI to remove all but one copy of the linkers at each end of the cDNA, and ligated to EcoRI-cut and dephosphorylated arms of bacteriophage λgt10 (Huynh et al., DNA Cloning: A Practical Approach, Glover, ed., IRL Press pp 49-78). The ligated DNA was packaged into phage particles to generate a library of 2.5×10^6 recombinants. 5×10^5 recombinants were plated on E. coli strain C600hf1$^-$ and screened by standard plaque hybridization techniques with the labeled oligonucleotide probes. Three positively hybridizing clones were isolated from the PBT library. These were plaque purified and used to prepare bacteriophage DNA which was digested with EcoRI. The digests were electrophoresed on an agarose gel, blotted onto nylon filters, and retested for hybridization of the fragments to the two oligonucleotide probes. One clone contained a DNA segment which positively hybridized to both probes. This DNA segment, containing an internal EcoRI cleavage site, was isolated by partial digestion with EcoRI followed by preparative agarose gel electrophoresis, then subcloned into an EcoRI-cut derivative of the standard cloning vector pBR322 (pGembl) containing a polylinker having a unique EcoRI site, a BamH1 site and numerous other unique restriction sites. The resulting plasmid was designated pGembl:hIL-4. An exemplary vector substantially similar to pGembl is described by Dente et al., Nucleic Acids Research 11:1645 (1983).

Following transformation of a suitable E. coli host strain, plasmid DNA was purified by standard techniques, then cut with EcoRV and BamH1. The resulting fragment was ligated to Asp718 and BamH1-cut pBC(CSF-1) and the following linker fragment, which provides the KEX2 α-factor processing site and the initial four amino acids of hIL-4 having a His^{25} amino terminus:

```
GTA CCT TTG GAT AAA AGA CAC AAG TGC GAT
    GA AAC CTA TTT TCT GTG TTC ACG CTA
       Leu Asp Lys Arg His Lys Cys Asp
```

The KEX2 protease cleaves the peptide immediately following the Arg residue. This construct was designated pBC104.

In substantially similar fashion, pBC103 was prepared by ligating the hIL-4 EcoRV-BamH1 fragment, Asp718 and BamH1-cut pBC(CSF-1), and the following oligonucleotide fragment, which encodes an additional His-Gly located immediately following the KEX2 cleavage site and preceding His^{25}:

```
GTA CCT TTG GAT AAA AGA CAC GGA CAC AAG TGC GAT
    GA AAC CTA TTT TCT GTG CCT GTG TTC ACG CTA
       Leu Asp Lys Arg His Gly His Lys Cys Asp
```

The resulting expression vectors, designated pBC103 and pBC104, were amplified in E. coli and then employed to transform yeast strain XV2181 by the procedures previously referenced. The transformed yeast were grown in nutrient media under conditions promoting derepression of the ADH2 promoter, and the resulting conditioned medium assayed for hIL-4 activity using goat anti-human IgM $F(ab)_2$ fragments as coactivator. These assays indicated a medium activity of 43,427 U/ml for media conditioned by pBC104-transformed XV2181, and 46,149 U/ml for media conditioned by pBC103-transformed XV2181.

Example 2: Construction of DNA Sequence Encoding Analog hIL-4

The two asparagine-linked glycosylation sites present in the natural hIL-4 protein (Asn^{62} and Asn^{129}) were removed by changing the codons at these positions to ones that encode aspartic acid. This

prevents N-linked glycosylation, or even hyperglycosylation, of the secreted protein by yeast cells, thereby enabling production of a more homogeneous product. The N-linked glycosylation sites in the hIL-4 cDNA described above (pBC104) were inactivated by replacing portions of the cDNA with synthetic oligonucleotides containing the desired nucleotide changes, as described below.

A cloning vector (pGembl:hIL-4) comprising the wild-type hIL-4 cDNA sequence shown in FIGURE 1 was digested with the restriction enzymes EcoRV, which cleaves after nucleotide 12 of mature hIL-4, and BamH1, which cleaves downstream from the hIL-4 cDNA in the polylinker region of the vector. The approximate 550 base pair hIL-4 cDNA fragment was subcloned into the pBR322-derived vector pPL-3 by digesting this vector with EcoRV and BamH1 (see FIGURE 3). The resulting plasmid was designated L225.

A DNA fragment from L225 containing the hIL-4 cDNA was then subcloned into the pBR322-derived vector pGEM-3 (Promega Biotec, Madison, WI, USA) by digesting plasmid L225 with ClaI (5′ of the hIL-4 cDNA), treating with T4 DNA polymerase to form blunt ends, then digesting with Sst1 (3′ to the hIL-4 cDNA in the polylinker region) to remove the cDNA-containing fragment. The vector pGEM-3 was digested with HindIII, treated with T4 DNA polymerase to form blunt ends, then digested with Sst1. The resulting plasmid was designated L257. This plasmid was used to perform the oligonucleotide replacement mutagenesis described below. All references to numbering of amino acid residues or nucleotides are in accordance with the numbering of FIGURE 1, in which residues and nucleotides are numbered from the N-terminus of the full length translation product, including the putative native signal peptide.

The codon encoding asparagine at position 62 was changed to a codon encoding aspartic acid as follows. Plasmid L257 was digested with HincII, which cuts at nucleotide 152, and PstI, which cuts at nucleotide 211. The resulting vector fragment was isolated and ligated to the following oligonucleotide A:

```
G ACG GTA ACC GAC ATC TTT GCT GCT AGC AAG GAC...
C TGC CAT TGG CTG TAG AAA CGA CGA TCG TTC CTG...
  Thr Val Thr Asp Ile Phe Ala Ala Ser Lys Asp

...ACA ACT GAG AAG GAA ACC TTC TGC A
...TGT TGA CTC TTC CTT TGG AAG
   Thr Thr Glu Lys Glu Thr Phe Cys.
```

The underlined nucleotides above represent changes from the wild type cDNA sequence. Only the A/T to G/C change at nucleotide 184 results in a codon specifying an amino acid change (Asn^{62} to Asp^{62}). The other five base changes do not alter the amino acid sequence, but introduce restriction sites (BstEII and NheI) to facilitate identification of the altered sequence.

The codon encoding the asparagine residue at position 129 was similarly changed to codon encoding aspartic acid by replacing the DNA fragment from the EcoR1 site (nucleotide 360) to the RsaI site (nucleotide 393) in the hIL-4 cDNA with the following synthetic oligonucleotide B:

```
AAT TCG TGT CCT GTG AAG GAA GCC GAC CAG TCG
     GG ACA GGA CAC TTC CTT CGG CTG GTC AGC
Asn Ser Cys Pro Val Lys Glu Ala Asp Gln Ser
```

The underlined nucleotides represent changes from the wild-type cDNA sequence. Only the A/T to G/C change at position 385 results in an codon specifying an amino acid change (Asn^{129} to Asp^{129}). The other base changes introduce a SalI restriction site without altering the amino acid sequence. The plasmid derived from plasmid L257 carrying both codon changes was designated pBC132.

Example 3: Construction of a Yeast Expression Vector for the hIL-4 Analog GluAlaGluAla-hIL-4-(Asp-62, Asp-129)

To prepare a yeast expression vector for the mutein, a DNA fragment encoding hIL-4(Asp^{62}, Asp^{129}) was removed from the pBC132 vector by digestion with EcoRV and SstI, manipulated as described below, and inserted into the yeast expression vector pIXY120. pIXY120 is substantially identical to pBC104, except for its heterologous insert. As noted below, pBC104 can be used in place of pIXY120 for expression of the muteins of the present invention.

The yeast expression vector pIXY120 (FIGURE 4) includes DNA sequences from the following sources:

1. From the E. coli vector pBR322, the large SphI (nucleotide 562) to EcoRI (nucleotide 4361) restriction fragment which includes the origin of replication and the ampicillin-resistance marker for selection in E. coli.

2. From the yeast S. cerevisiae, DNA fragments include the TRP-1 gene as a selectable marker in yeast; the yeast 2 micron origin of replication; and the S. cerevisiae ADH2 promoter; and an 85 amino acid signal peptide derived from the gene encoding the secreted peptide α-factor (See Brake et al., Proc. Natl. Acad. Sci. USA 81:4642 (1984); Kurjan and Herskowitz, Cell 30:933 (1982); and U.S. Patent 4,546,082). An Asp718 restriction site was introduced at nucleotide 237 in the α-factor signal peptide to facilitate its fusion to heterologous genes. The T residue at nucleotide 241 was changed to a C residue by oligonucleotide-directed in vitro mutagenesis.

3. A synthetic oligonucleotide containing multiple cloning sites was inserted from the Asp718 site (amino acid 79) near the 3′ end of the α-factor signal peptide to a Spe1 site contained in the 2μ sequences:

GTACCTTTGGATAAAAGAGACTACAAGGACGACGATGACAAGAGGCCTCCATGGATCCCCCGGGACA
GAAACCTATTTTCTCTGATGTTCCTGCTGCTACTGTTCTCCGGAGGTACCTAGGGGGCCCTGTGATC

4. A 514 bp DNA fragment derived from the single-stranded bacteriophage f1 containing the origin of replication and intergenic region. This fragment is inserted at the Nru1 site in the pBR322 DNA sequences. The presence of the f1 origin of replication allows generation of single-stranded copies of the vector when transformed into appropriate (male) strains of E. coli and superinfected with bacteriophage f1. This capability facilitates DNA sequencing of the vector and allows the possibility of doing in vitro mutagenesis.

The yeast expression vector pIXY120 was digested with the restriction enzyme Asp718, which cleaves near the 3′ end of the α-factor leader peptide (nucleotide 237), and BamHI, which cleaves in the polylinker. The large vector fragment was purified and ligated to the following two DNA fragments, as depicted in FIGURE 4:

1. The hIL-4 cDNA fragment from the EcoRV site (nucleotide 136 of mature hIL-4) to the BamHI site (3′ to the hIL-4 cDNA in the Gembl:hIL-4 polylinker) obtained from plasmid Gembl:hIL-4.

2. The following synthetic oligonucleotide linker 1, which regenerates the 3′ end of the α-factor leader peptide and fuses it in frame to the 5′ four amino acids of hIL-4. This oligonucleotide also encodes an eight amino acid identification peptide fused to the N-terminus of hIL-4. This fusion to the hIL-4 protein allowed its detection with specific antibody and was used initially for monitoring the expression and purification of hIL-4.

```
GTA CCT TTG GAT AAA AGA GAC TAC AAG GAC GAC GAT GAC AAG CAC AAG TGC GAT
     GA AAC CTA TTT TCT CTG ATG TTC CTG CTG CTA CTG TTC GTG TTC ACG CTA
   Pro Leu Asp Lys Arg Asp Tyr Lys Asp Asp Asp Asp Lys His Lys Cys Asp
 ←--------α-factor-----→|←---identification peptide-----→|←-----hIL-4---→
```

This plasmid, designated pIXY118 (FIGURE 5) contains the wild type hIL-4 gene under control of the glucose repressible ADH2 promoter. The α-factor leader peptide allows secretion of hIL-4 from the yeast cells. Proteolytic processing of the α-factor leader occurs after the Lys-Arg residues (amino acids 83 and 84) of the α-factor leader.

To create a yeast expression vector containing the hIL-4 gene without the consensus N-linked glycosylation sites, plasmid pIXY 118 was digested with EcoRI, which cleaves 5′ to the ADH2 promoter, and SstI, which cleaves 3′ to the hIL-4 gene (FIGURE 5). The large vector fragment was purified and ligated to the following DNA fragments:

1. The EcoRI to EcoRV DNA fragment from pIXY118 carrying the ADH2 promoter, the α-factor leader sequences and the first four amino acids of hIL-4 (this was necessary because of an SstI site in this fragment).

2. The hIL-4 cDNA insert contained in plasmid pBC132 from the EcoRV site (from nucleotide 13 of mature hIL-4) to the SstI site (3′ to the hIL-4 cDNA).

The resulting plasmid was designated pIXY133. It contained the hIL-4 gene with the Asp^{62} and Asp^{129} codon changes and the eight

amino acid fusion peptide at the N-terminus in the yeast expression vector.

The final yeast expression plasmid is identical to plasmid pIXY133 except for the oligonucleotide linker sequences used to fuse the hIL-4 cDNA to the α-factor leader (oligonucleotide 2, FIGURE 6). This yeast expression plasmid was constructed as described below and shown in FIGURE 6:

The yeast expression vector pIXY120 was cleaved with the restriction enzymes Asp718 and BamHI as described above. The large vector fragment was ligated together with the following DNA fragments: (1) a hIL-4 (Asp^{62} Asp^{129}) cDNA fragment derived from plasmid pIXY133 from EcoRV (at nucleotide 13) to the BamHI site (3′ to the hIL-4 cDNA) and (2) a synthetic oligonucleotide (oligonucleotide 2, FIGURE 6) regenerating the 3′ end of the α-factor leader peptide from the Asp718 site (the amino acids Pro-Leu-Asp-Lys-Arg-Glu-Ala-Glu-Ala) and fusing it in-frame to the N-terminal four amino acids of hIL-4 to the EcoRV site. The sequence of this oligonucleotide is set forth below:

```
GTA CCT TTG GAT AAA AGA GAA GCT GAA GCT CAC AAG TGC GAT
    GA AAC CTA TTT TCT CTT CGA CTT CGA GTG TTC ACG CTA
  Pro Leu Asp Lys Arg Glu Ala Glu Ala His Lys Cys Asp
←---α-factor--------→|←--------hIL-4 analog---------→
```

The resulting plasmid was designated pIXY157 (FIGURE 6). This vector, when present in yeast, allows glucose-regulated expression and secretion of a non-glycosylated mutant hIL-4. The hIL-4 that is recovered contains the four amino acids Glu-Ala-Glu-Ala at the N-terminus due to lack of processing by the yeast protease dipeptidyl-amino-peptidase A. The large portion of the α-factor leader is proteolytically removed after the Lys-Arg residues (amino acids 83 and 84 of the leader) by the product of the KEX2 gene.

The foregoing rather lengthy route can be shortcut by excising an EcoRV-BamHI IL-4 cDNA-containing fragment from pBC104, and digesting the fragment and reassembling it as an EcoRV-SstI fragment as described above using synthetic oligonucleotides to alter the asparagine-linked glycosylation sites. pBC104 can also be cut with EcoRI and SstI, and with EcoRI and EcoRV as described above for

pIXY118, to generate vector EcoRI-SstI and EcoRI-EcoRV fragments which can be ligated together with the reassembled mutant IL-4 EcoRV-SstI fragment. This construct can then be cut with Asp718 and BamHI and the resulting vector fragment ligated to (1) an EcoRV-BamHI fragment from the same plasmid comprising the IL-4 analog gene, and (2) the foregoing synthetic oligonucleotide 2, to generate a yeast expression vector for GluAlaGluAla-hIL-4(Asp^{62}, Asp^{129}) which is identical to pIXY157.

Example 4: Fermentation of Yeast and Analog Protein Purification

Yeast containing the expression plasmid encoding the hIL-4 analog protein GluAlaGluAla-hIL-4-(Asp^{62}, Asp^{129}) were maintained on YNB-trp agar plates stored at 4°C. New plates were prepared from frozen glycerol stocks (-70°C) once a week.

A preculture was started by inoculating several isolated recombinant yeast colonies into one liter of YNB-trp medium (6.7 g/L Yeast Nitrogen Base, 5 g/L casamino acids, 40 mg/L adenine, 160 mg/L uracil, and 200 mg/L tyrosine), and grown overnight in two 2-liter flasks at 30°C with vigorous shaking. By morning the culture was saturated, in stationary phase, at an OD600 of 2 to 7.

Two 10 liter fermenters were cleaned and sterilized, then filled to 80% of their working capacity with 12/50 YEP medium (12 g/L yeast extract, 50 g/L peptone) and maintained at 30°C with 500-600 rpm agitation and 10-16 LPM aeration. The inoculum was added. After two hours of growth a nutrient feed of 50% glucose was begun at a rate such that 50 g/L is added over a period of 10-12 hours. The nutrient feed was then shifted to 50% ethanol added to a total of 10 ml/L over 6 hours.

Total elapsed time of fermentation was approximately 20 hours. The final optical density (600nm) ranged from 30 to 45. The fermenters were cooled to 20°C, and the harvesting procedure begun. First, the pH was adjusted to 8.0 by the addition of 5M NaOH. The fermenter contents were harvested into a clean carboy. The yeast beer was then filtered through a Millipore Pellicon filter system equipped with a 0.45 micron filter cassette, and collected in a sterile 10 L carboy.

The GluAlaGluAla-hIL-4(Asp^{62}, Asp^{129}) mutein (IL-4 mutein) in the filtered yeast supernatants was purified by batch absorption on S-Sepharose gel, washing with 50mM β-alanine pH 4.0 and 50mM HEPES pH 7.4, elution with a solution of 0.5M NaCl and 50mM HEPES pH 7.4, high performance liquid chromatography (HPLC), application to a MONO-S column, and dialysis against 100mM Tris.

In the first step, the IL-4 mutein contained in the yeast beer was bound to S-Sepharose gel by batch absorption. In a typical run, 400 ml of S-Sepharose gel slurry (1 volume gel:1 volume 0.5M β-alanine pH 4.0) was added to a volume of 10 L of yeast beer. The pH of this solution was adjusted to pH 3.6 by adding 2N HCl. The solution was then stirred for 10 minutes, and the gel allowed to settle for 30 minutes. The supernatant was decanted through a sintered glass funnel, and the gel slurry containing the recombinant hIL-4 mutein was retained in the funnel.

The gel was washed with 500 ml of 50mM β-alanine pH 4.0, followed by two 1 L washes with 50mM HEPES pH 7.4. The IL-4 mutein is then eluted from the gel by five 200 ml washes with a solution of 0.5M NaCl and 50mM HEPES pH 7.4. S-Sepharose elutions 1 through 3, containing the highest concentrations of the IL-4 mutein, were pooled, sterile filtered, and stored at 4°C until HPLC processing. Elutions from the 4th and 5th washes, containing <10% of the IL-4 mutein, were pooled separately, sterile filtered, and stored at 4°C. Samples from the crude yeast beer, unbound fraction, each of the three washes, and eluate from pooled fractions 1-3 and 4-5 were tested for the presence of IL-4 by immunodot blot and SDS-PAGE. Protein concentration in the pooled eluates was determined by BCA Assay. S-Sepharose fractions were collected until 100 L of yeast beer were processed. At that time, a pool of all elutions from washes 1-3 (as described above) was applied to a 5cm x 30cm column packed with 15-20μ C-4 reversed phase silica using the Waters LC-500 HPLC equilibrated in 0.1% trifluoroacetic acid (TFA)/pyrogen free water. The C-4 column was washed with 1 L of a solution of 0.1% TFA/pyrogen free water. The fractions containing recombinant hIL-4 were then applied to the column and eluted with a gradient of 0.1% TFA in acetonitrile at a rate of change of 2% per minute and a flow rate of 100 ml per minute.

Peak fractions from C-4 RPC column were pooled and 1/10 volume of 0.5M β-alanine pH 4 was added. A sample was taken and then the pool was applied to a 20 ml MONO-S column (1.6 cm x 10 cm, Pharmacia) at 7 ml/minute. After sample application, the column was washed with 100 ml of 50mM Tris pH 7.4, and the IL-4 mutein was eluted with a linear gradient of 1 M NaCl, 100mM Tris pH 8. Peak fractions of IL-4 were then pooled and dialyzed against 100mM Tris pH 8 overnight at 4°C, then sterile filtered. Upon completion of manufacturing and purification, the total bulk active product was pooled and stored in sterile polyethylene tubes at 4°C. The specific activity of the purified hIL-4(Asp^{62}, Asp^{129}) by the BCGF assay was 3.1 ± 10^7 units per mg.

Example 5: Induction of Cytolytic Activity in Mixed Leukocyte Culture

IL-4 has been shown to stimulate proliferation of certain factor-dependent, non-B lineage cell lines that are normally responsive to IL-2 or to the myeloid growth factor, IL-3. See Grabstein et al., J. Exp. Med. 163:1405 (1986) and Lee et al., Proc. Natl. Acad. Sci. USA 83:2061 (1986). To demonstrate that IL-4 also affects primary T cell populations, particularly with regard to the generation of functionally active T cells, its effects on the generation of CTL in mixed leukocyte cultures (MLC) were assessed. MLC were established with a suboptimal concentration of C57BL/6 splenic responding cells and allogeneic, irradiated DBA/2 splenic stimulating cells. Five days after culture initiation, lytic activity against ^{51}Cr-labeled P815 murine (DBA/2 origin) tumor target cells was measured.

Murine IL-4 cDNA was cloned from a library made from sized mRNA of phorbol myristate acetate stimulated EL4 thymoma cells using the cDNA sequence published by Lee et al., Proc. Natl. Acad. Sci. USA 83:2061 (1986). A full length cDNA was subcloned into a yeast expression vector which included pBR322 sequences, the TRP1 gene of yeast for tryptophan selection, the yeast 2μ origin of replication and the yeast alcohol dehydrogenase 2 (ADH2) promoter and α-factor leader sequences sufficient to direct synthesis and secretion. The expression plasmid was transformed into yeast strain 79 (α, trp1-1,

leu2-2) selecting for Trp^+ transformants. Cultures were grown for purification by inoculating 1 liter of rich medium (1% yeast extract, 2% peptone, 2% glucose) and growing the cultures at 30°C to stationary phase. PMSF and pepstatin A were added at the time of harvest. Cells were removed by centrifugation and filtration through a 0.45 µm cellulose acetate filter. rIL-4 was purified to homogeneity from yeast supernatant by five cycles of high performance liquid chromatography (HPLC) using solvent systems previously described by Urdal et al., J. Chromatography 296:171 (1984). Homogeneous recombinant and natural murine IL-4 exhibit identical specific activities of 2.0×10^5 U/µg, as measured in the B cell proliferation assay described below.

MLC incorporating 5×10^5 C57BL/6 murine spleen cells and 5×10^6 gamma irradiated (2,500r) DBA/2 murine splenic stimulating cells were initiated in 16 mm diameter culture wells containing 2 ml culture medium. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal bovine serum (FBS), 5×10^{-5} M 2-mercaptoethanol and additional amino acids, substantially as disclosed by Cerottini et al., J. Exp. Med. 140:703 (1974). Cultures were supplemented at initiation with homogeneous natural murine IL-4 (nIL-4) at 2 ng/ml, recombinant human IL-2 at 10 ng/ml, or medium. Five days after culture initiation, lytic activity was tested by incubating, in duplicate 200 µl volumes, varying ratios of effector cells and ^{51}Cr labeled P815 target cells (2×10^3 cells/well) in 96 well v-bottom microtiter plates. After a 3.5 hr incubation, plates were centrifuged and 150 µl supernatant from each well were harvested and counted in a gamma counter. The results obtained are indicated in FIG. 6. In FIG. 6, reported percent specific ^{51}Cr release was determined as 100 x [cpm (experimental) - cpm (spontaneous)] / [cpm (maximum) - cpm (spontaneous)] where spontaneous release (118 cpm) was determined by incubating P815 in medium and maximum release (801 cpm) by incubating P815 in 1N HCl. One lytic unit (LU) was defined as the number of cells required to achieve 50% lysis of 2×10^3 P815 target cells and is determined from the dose-response curve. Percent recovery equals the number of cells recovered at day 5 as a percentage of the initial number of responding cells cultured.

Cultures supplemented at initiation with 2 ng/ml of homogeneous, natural IL-4 exhibited approximately 50-fold greater cytolytic activity, on a per cell basis, than control cultures in which exogenous IL-4 was not present, and 100-fold more activity on a per culture basis. Cultures supplemented with 10 ng/ml rIL-2, as expected, also exhibited higher levels of CTL activity than control cultures, but the lytic activity was 7-fold less than that which developed in IL-4 supplemented cultures. Cytolytic T lymphocyte generated in MLC supplemented with either IL-4 or IL-2 were alloantigen specific, since lytic activity directed against target cells syngeneic with the responding cell populations was < 2% of that directed against the specific allogeneic target (data not shown). These data indicate that IL-4 is a potent helper factor for the generation of alloreactive cytolytic T lymphocytes.

Example 6:
Induction of Cytolytic Activity in Memory T Cell Populations

MLC populations that have been cultured for extended periods of time gradually lose CTL activity but can be re-induced to express high level cytolytic activity by exposure to either allogeneic cells or culture supernatant. To test the effects of IL-4 on such MLC memory populations, cells obtained from day 14 C57BL/6 anti-DBA/2 primary MLC were cultured in the presence of recombinant IL-2 or IL-4 and resultant cytolytic activity was measured three days later.

Mixed leukocyte cultures were established with 25×10^6 C57BL/6 spleen cells and 25×10^6 irradiated (2500r) DBA/2 splenic stimulating cells in 25 cm^2 flasks, 20 ml total volume. Fourteen days after initiation, cells were harvested from primary cultures and 5×10^5 cells were re-cultured in Costar 16 mm culture wells in 2 ml volumes containing recombinant murine IL-4 at 1 ng/ml, recombinant human IL-2 at 0.5 ng/ml, or medium. Three days later, culture contents were tested for lytic activity against ^{51}Cr-labeled P815 target cells. Spontaneous release in this experiment was 204 cpm, while maximum release was 1,829 cpm.

As shown in FIG. 7, exposure of the cells to either lymphokine resulted in cellular proliferation and induction of high

level cytolytic activity. Lytic activity generated in cultures incubated with IL-4 was approximately 100 fold higher than that observed in control cultures incubated in medium (FIG. 7), and 80-fold higher than the activity of the day 14 population before exposure to exogenous lymphokine (data not shown). CTL activity induced by IL-4 in these cultures, as in primary MLC, was antigen-specific (data not shown). Thus, IL-4, like IL-2, induces expression of antigen-specific cytolytic activity in once-activated, resting memory T cell populations without the need for further antigenic stimulation.

Example 7: Dose-Response Comparison of IL-4 and IL-2

To test directly the relative efficiencies of recombinant IL-4 and IL-2 in their capacity to augment CTL generation, multiple concentrations of homogeneous recombinant IL-4 or IL-2 were added to allogeneic primary mixed leukocyte cultures and resultant lytic activity was measured five days later.

In this experiment, mixed leukocyte cultures (MLC) were established with 2×10^6 C57BL/6 spleen cells and 5×10^6 irradiated (2500r) spleen cells from either DBA/2 (allogeneic) or C57BL/6 (syngeneic) and supplemented with varying doses of recombinant IL-4 or IL-2. Lytic activity against ^{51}Cr labeled P815 was assessed on day 5, as above. Spontaneous release of radiolabel averaged 125 cpm, while the maximum release observed was 886 cpm.

Although both lymphokines augmented cell proliferation and CTL activity, the levels of lytic activity that developed in cultures containing optimal doses of IL-4 were approximately 3-4 fold higher than that observed in cultures supplemented with optimal doses of IL-2. In addition, at suboptimal lymphokine doses, approximately 10-fold less IL-4 than IL-2 was required to obtain equivalent amounts of lytic activity. These data indicate that, in mixed leukocyte cultures established with this allogeneic strain combination, IL-4 is a more potent helper factor for generating CTL from unprimed precursors than IL-2. Since approximately equal numbers of cells were recovered in allogeneic MLC supplemented with IL-2 or IL-4, the data may reflect either higher CTL frequency or individual CTL with greater lytic activity.

Table 2: Effects of IL-4 and IL-2 on generation of cytolytic activity in allogeneic and syngeneic primary mixed leukocyte culture (MLC).

Culture Supplement	A. Allogeneic MLC % Recovery	A. Allogeneic MLC LU/Culture	B. Syngeneic MLC % Recovery	B. Syngeneic MLC LU/Culture
Medium	55	4	21	<1
rIL-2,ng/ml				
10^3	101	23	185	44
10^2	99	30	119	37
10	90	37	48	4
1	61	12	33	<2
10^{-1}	73	7	22	<1
10^{-2}	79	6	30	<1
rIL-4,ng/ml				
10^2	85	95	18	<1
10	65	124	15	<1
1	63	79	17	<1
10^{-1}	53	12	20	<1
10^{-2}	66	6	16	<1

The data presented above and in Examples 6 and 7 demonstrate that IL-4 induces both proliferation and cytolytic activity in primary and memory MLC populations, revealing a novel regulatory mechanism for CTL generation.

Example 8: Induction of Thymocyte proliferation

Thymocytes were obtained from female C57BL/6J mice, 6-10 weeks of age, and cultured at 1.5×10^6 cells/well in 200 µl volumes of RPMI 1640 containing 5% FBS, culture supplements as described above, and in the presence or absence of 0.25% PHA-M (Gibco Laboratories, Grand Island, NY) and either recombinant human IL-2 or murine IL-4, as indicated in Table 3, below. Cultures were pulsed with 2.0 µCi of [^{3}H]thymidine (75 Ci/mmole) during the last 18 hours of a 72 hour culture period, harvested onto glass fiber filters and incorporated radioactivity determined.

Both rIL-2 and rIL-4 were used at 10 ng/ml. Results are expressed in Table 3, below, as the mean cpm (± the standard deviation) of triplicate cultures.

Table 3: Stimulation of thymocyte proliferation by rIL-4

Culture Additive	cpm (± s.d.)
none	320 (41)
rIL-4	11,183 (639)
rIL-2	60,014 (5707)
PHA	2,050 (184)
PHA + rIL-4	83,162 (7548)
PHA + rIL-2	138,955 (9019)

These results indicate that IL-4, in the presence and absence of a comitogenic stimulus, induces proliferation of thymocytes. In the presence of added mitogen, proliferation is considerably (7x) enhanced.

Example 9: Stimulation of Memory T Cell Proliferation by IL-4

Memory T cells were generated in 14 day primary MLC. Primary MLC were established with 25 x 10^6 C57BL/6 spleen cells and 25 x 10^6 irradiated (2500r) DBA/2 splenic stimulating cells in 25 cm^2 flasks containing 20 ml of culture medium. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM) containing 2% fetal bovine serum (FBS), 5 x 10^{-5} M 2- mercaptoethanol and additional amino acids, substantially as disclosed by Cerottini et al., J. Exp. Med. 140:703 (1974). For secondary MLC, 5 x 10^5 cells recovered from day 13 primary MLC were cultured with 5 x 10^6 irradiated (2500r) DBA/2 spleen cells in 16 mm culture wells containing 2 ml culture medium. These cells were then tested for proliferation in response to rIL-4 (4 ng/ml) or rIL-2 (4 ng/ml) either before or three days after restimulation with allogeneic cells, by incubating 10^4 cells/well in 96 well flat bottom plates containing 200 µl/well culture medium and the indicated additive. Cultures were pulsed for the last 18 hours of a 72 hour culture period with 1.0 µCi of [^{3}H]thymidine (75 Ci/mmole, New England Nuclear, Boston, MA) and then harvested onto glass fiber filters. Incorporation of radioactivity was measured by liquid

scintillation spectrometry. Results are expressed in Table 4, below, as the mean cpm (± the standard deviation) of triplicate cultures.

Table 4: Response to rIL-4 of Resting and Activated Memory T cells

Culture Additive	Day 14 Primary MLC	Day 3 Secondary MLC
none	497 (426)	631 (253)
rIL-4	2,422 (161)	61,246 (3895)
rIL-2	32,871 (4051)	36,776 (3971)

The foregoing results indicate a distinction between the proliferation-inducing effects of IL-4 and IL-2. Unlike IL-4, IL-2 is capable of inducing proliferation of cells late in the culture cycle without reactivation by antigen. However, when restimulated by alloantigen memory cells are significantly more responsive to added IL-4 than added IL-2.

CLAIMS

What is claimed is:

1. A human interleukin-4 (hIL-4) analog protein comprising at least one amino acid substitution, deletion, or insertion resulting in inactivation of a asparagine-linked glycosylation site, deletion or substitution of a cysteine residue, or modification of a yeast KEX2 protease recognition site.

2. An hIL-4 analog according to Claim 1, having a mutant amino acid sequence which is substantially homologous to the wild-type amino acid sequence of hIL-4, wherein at least one occurrence Asn-A^1-Z in the wild-type sequence has been replaced in the mutant sequence by Asn-A^2-Y or X-A^2-A^3, where

A^1, A^2, and A^3 are the same or different and can be any amino acid,

X is any amino acid not Asn;

Y is any amino acid not Z; and

Z is Ser or Thr.

3. An hIL-4 analog protein according to Claim 2, hIL-4(Asp^{62}, Asp^{129}).

4. An hIL-4 analog protein according to Claim 3, GluAlaGluAla-hIL-4(Asp^{62}, Asp^{129}).

5. A DNA sequence encoding an hIL-4 analog protein according to any of Claims 1-4.

6. A recombinant expression vector comprising a DNA sequence according to Claim 5.

7. A process for preparing an hIL-4 analog protein, comprising culturing a microorganism transformed with a recombinant expression vector according to Claim 6 under conditions promoting expression.

8. A pharmaceutical composition for inducing proliferation of and lytic activity in a population of antitumor cytolytic T lymphocytes, comprising contacting T cells with a composition comprising a biologically effective quantity of IL-4 or an analog hIL-4 according to any of Claims 1-4 in combination with a physiologically acceptable carrier or diluent.

9. A method for inducing proliferation of and lytic activity in a population of cytolytic T lymphocytes, comprising contacting T cells with a composition comprising a biologically effective quantity of IL-4 or an analog hIL-4 according to any of Claims 1-4 in combination with a physiologically acceptable carrier or diluent.

10. A method according to Claim 9, wherein the lymphocytes are previously activated by exposure to virus-associated antigen.

11. A method according to Claim 9, wherein the lymphocytes are previously activated by exposure to tumor antigen.

12. A method according to Claim 9, wherein the lymphocytes are induced and expanded ex vivo and readministered to a patient in adoptive immunotherapy.

13. A method for inducing proliferation and activation of antitumor or antiviral cytolytic T lymphocytes in a mammal, comprising administering a therapeutically effective amount of IL-4 or an analog hIL-4 according to any of Claims 1-4.

14. An antiviral composition comprising a biologically effective amount of IL-4 or an analog hIL-4 according to any of Claims 1-4 and a physiologically acceptable carrier or diluent.

15. An antitumor composition comprising a biologically effective amount of IL-4 or an analog hIL-4 according to any of Claims 1-4 and a physiologically acceptable carrier or diluent.

16. An antitumor composition according to Claim 15, additionally comprising a therapeutically effective quantity of T lymphocytes expanded ex vivo in the presence of IL-4 or IL-2.

FIG. 1: Sequence of Native hIL-4

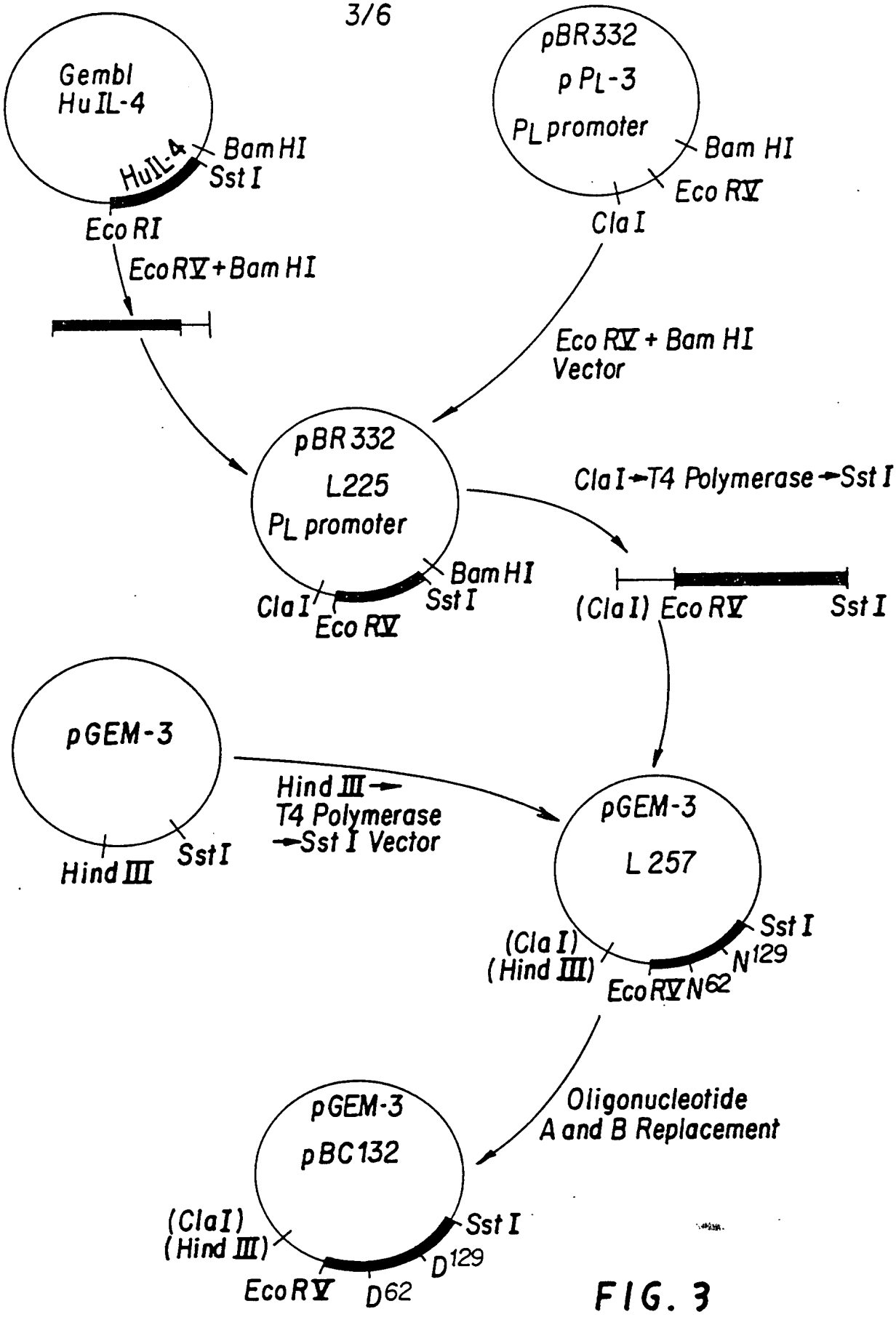
```
ATG GGT CTC ACC TCC CAA CTG CTT CCC CCT CTG TTC TTC CTG CTA   45
Met Gly Leu Thr Ser Gln Leu Leu Pro Pro Leu Phe Phe Leu Leu   15

GCA TGT GCC GGC AAC TTT GTC CAC GGA CAC AAG TGC GAT ATC ACC   90
Ala Cys Ala Gly Asn Phe Val His Gly His Lys Cys Asp Ile Thr   30

TTA CAG GAG ATC ATC AAA ACT TTG AAC AGC CTC ACA GAG CAG AAG  135
Leu Gln Glu Ile Ile Lys Thr Leu Asn Ser Leu Thr Glu Gln Lys   45

ACT CTG TGC ACC GAG TTG ACC GTA ACA GAC ATC TTT GCT GCC TCC  180
Thr Leu Cys Thr Glu Leu Thr Val Thr Asp Ile Phe Ala Ala Ser   60

AAG AAC ACA ACT GAG AAG GAA ACC TTC TGC AGG GCT GCG ACT GTG  225
Lys Asn Thr Thr Glu Lys Glu Thr Phe Cys Arg Ala Ala Thr Val   75

CTC CGG CAG TTC TAC AGC CAC CAT GAG AAG GAC ACT CGC TGC CTG  270
Leu Arg Gln Phe Tyr Ser His His Glu Lys Asp Thr Arg Cys Leu   90

GGT GCG ACT GCA CAG CAG TTC CAC AGG CAC AAG CAG CTG ATC CGA  315
Gly Ala Thr Ala Gln Gln Phe His Arg His Lys Gln Leu Ile Arg  105

TTC CTG AAA CGG CTC GAC AGG AAC CTC TGG GGC CTG GCG GGC TTG  360
Phe Leu Lys Arg Leu Asp Arg Asn Leu Trp Gly Leu Ala Gly Leu  120

AAT TCC TGT CCT GTG AAG GAA GCC AAC CAG AGT ACG TTG GAA AAC  405
Asn Ser Cys Pro Val Lys Glu Ala Asn Gln Ser Thr Leu Glu Asn  135

TTC TTG GAA AGG CTA AAG ACG ATC ATG AGA GAG AAA TAT TCA AAG  450
Phe Leu Glu Arg Leu Lys Thr Ile Met Arg Glu Lys Tyr Ser Lys  150

TGT TCG AGC TGA                                             495
Cys Ser Ser End                                             153
```

FIG. 1

FIG. 2: Sequence of GluAlaGluAla-hIL-4(Asp62, Asp129)

```
GTA

←---α-factor------→|←-----------hIL-4 mutein-------→
CCT TTA GAT AAA AGA GAA GCT GAA GCT CAC AAG TGC CAT ATC ACC   90
Pro Leu Asp Lys Arg Glu Ala Glu Ala His Lys Cys Asp Ile Thr   30

TTA CAG GAG ATC ATC AAA ACT TTG AAC AGC CTC ACA GAG CAG AAG  135
Leu Gln Glu Ile Ile Lys Thr Leu Asn Ser Leu Thr Glu Gln Lys   45

                        ↓BstEII                      NheI
ACT CTG TGC ACC GAG TTG ACG GTA ACC GAC ATC TTT GCT GCT AGC  180
Thr Leu Cys Thr Glu Leu Thr Val Thr Asp Ile Phe Ala Ala Ser   60

                                          ↓PstI
AAG GAC ACA ACT GAG AAG GAA ACC TTC TGC AGG GCT GCG ACT GTG  225
Lys Asp Thr Thr Glu Lys Glu Thr Phe Cys Arg Ala Ala Thr Val   75

CTC CGG CAG TTC TAC AGC CAC CAT GAG AAG GAC ACT CGC TGC CTG  270
Leu Arg Gln Phe Tyr Ser His His Glu Lys Asp Thr Arg Cys Leu   90
 52                      58                          66
GGT GCG ACT GCA CAG CAG TTC CAC AGG CAC AAG CAG CTG ATC CGA  315
Gly Ala Thr Ala Gln Gln Phe His Arg His Lys Gln Leu Ile Arg  105

TTC CTG AAA CGG CTC GAC AGG AAC CTC TGG GGC CTG GCG GGC TTG  360
Phe Leu Lys Arg Leu Asp Arg Asn Leu Trp Gly Leu Ala Gly Leu  120
 82                                                    96
↓EcoRI                                  ↓SalI
AAT TCC TGT CCT GTG AAG GAA GCC CAC CAG TCG ACG TTG GAA AAC  405
Asn Ser Cys Pro Val Lys Glu Ala Asp Gln Ser Thr Leu Glu Asn  135

TTC TTG GAA AGG CTA AAG ACG ATC ATG AGA GAG AAA TAT TCA AAG  450
Phe Leu Glu Arg Leu Lys Thr Ile Met Arg Glu Lys Tyr Ser Lys  150

TGT TCG AGC TGA                                             495
Cys Ser Ser End                                             153
```

FIG. 2C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HuIL-4
Bam HI
Sst I
Eco RI
Eco RV + Bam HI
pBR332
p PL-3
PL promoter
Bam HI
Eco RV
Cla I
Eco RV + Bam HI
Vector
pBR332
L225
PL promoter
Bam HI
Cla I
Sst I
Eco RV
Cla I → T4 Polymerase → Sst I
(Cla I) Eco RV
Sst I
pGEM-3
Hind III
Sst I
Hind III → T4 Polymerase → Sst I Vector
pGEM-3
L 257
Sst I
(Cla I)
(Hind III)
N129
Eco RV N62
Oligonucleotide
A and B Replacement
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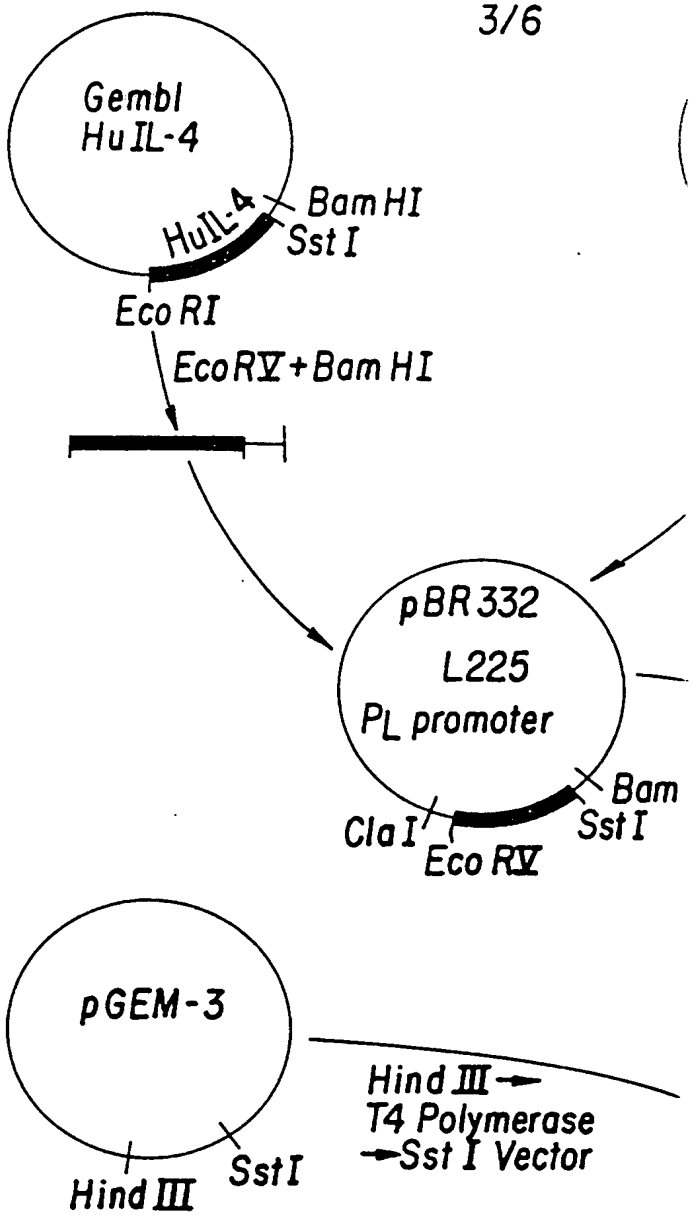
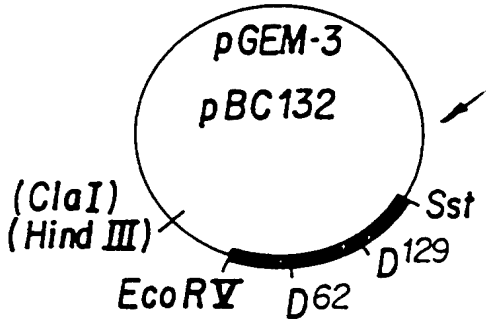
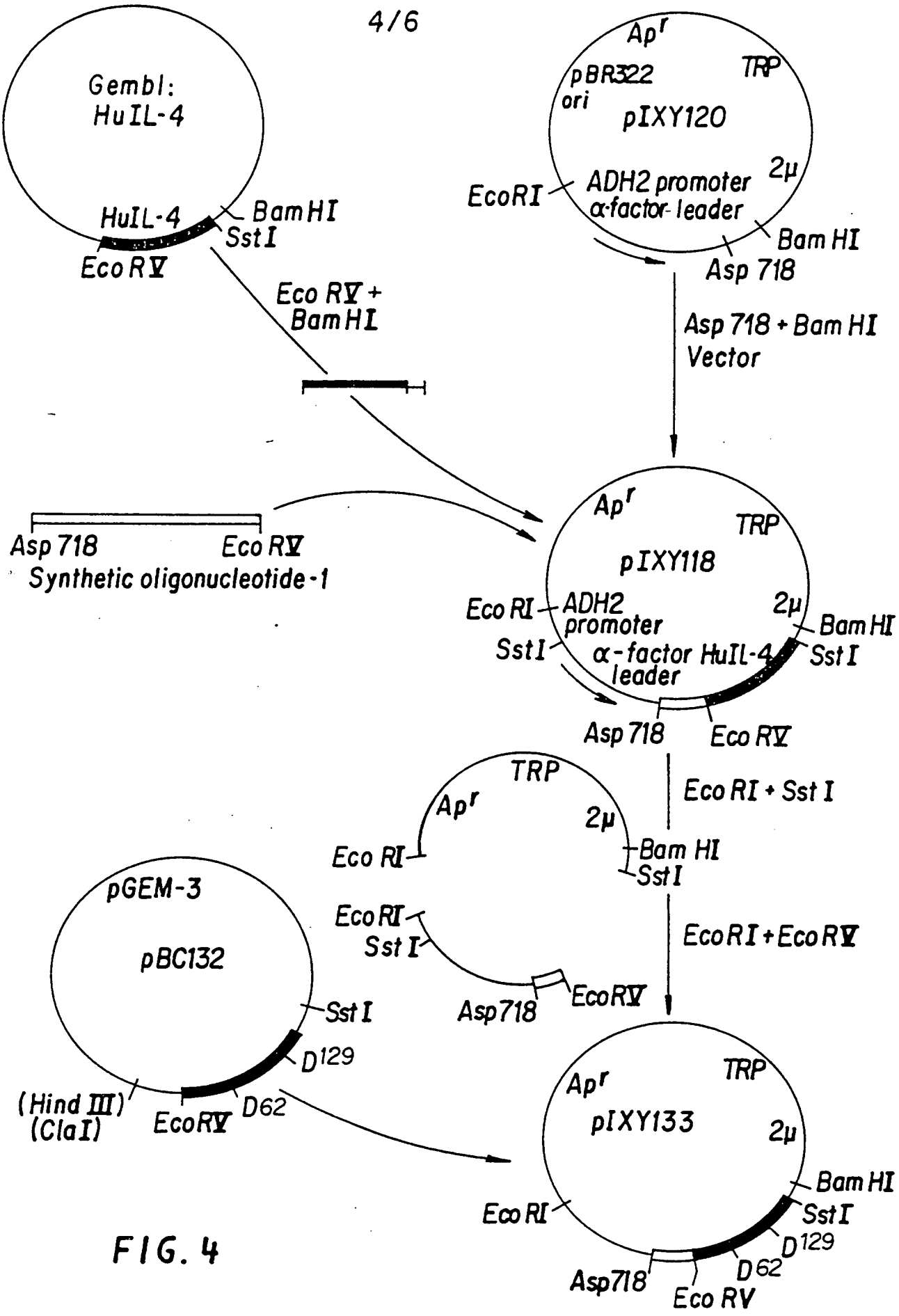
pBC132
(Cla I)
(Hind III)
Sst I
D129
Eco RV
D62

FIG. 3


GemblHuIL-4
HuIL-4
Bam HI
Sst I
Eco RI
EcoRV + Bam HI
pBR 332
L225
PL promoter
Bam
Sst I
Cla I
Eco RV
pGEM-3
Hind III
T4 Polymerase
Sst I Vector
Sst I
Hind III


pGEM-3
pBC132
(ClaI)
(Hind III)
Sst
D129
EcoRV
D62


Gembl:
HuIL-4
HuIL-4
Bam HI
Sst I
Eco RV
Eco RV +
Bam HI
Asp 718
Eco RV
Synthetic oligonucleotide-1
Ap^r
pBR322
ori
TRP
pIXY120
2μ
EcoRI
ADH2 promoter
α-factor leader
Bam HI
Asp 718
Asp 718 + Bam HI
Vector
Ap^r
TRP
pIXY118
Eco RI
ADH2
promoter
2μ
Bam HI
Sst I
α-factor
leader
HuIL-4
Sst I
Asp 718
Eco RV
Eco RI + Sst I
TRP
Ap^r
2μ
Eco RI
Bam HI
Sst I
EcoRI
Sst I
Asp718
EcoRV
EcoRI + EcoRV
pGEM-3
pBC132
Sst I
D129
(Hind III)
(ClaI)
EcoRV
D62
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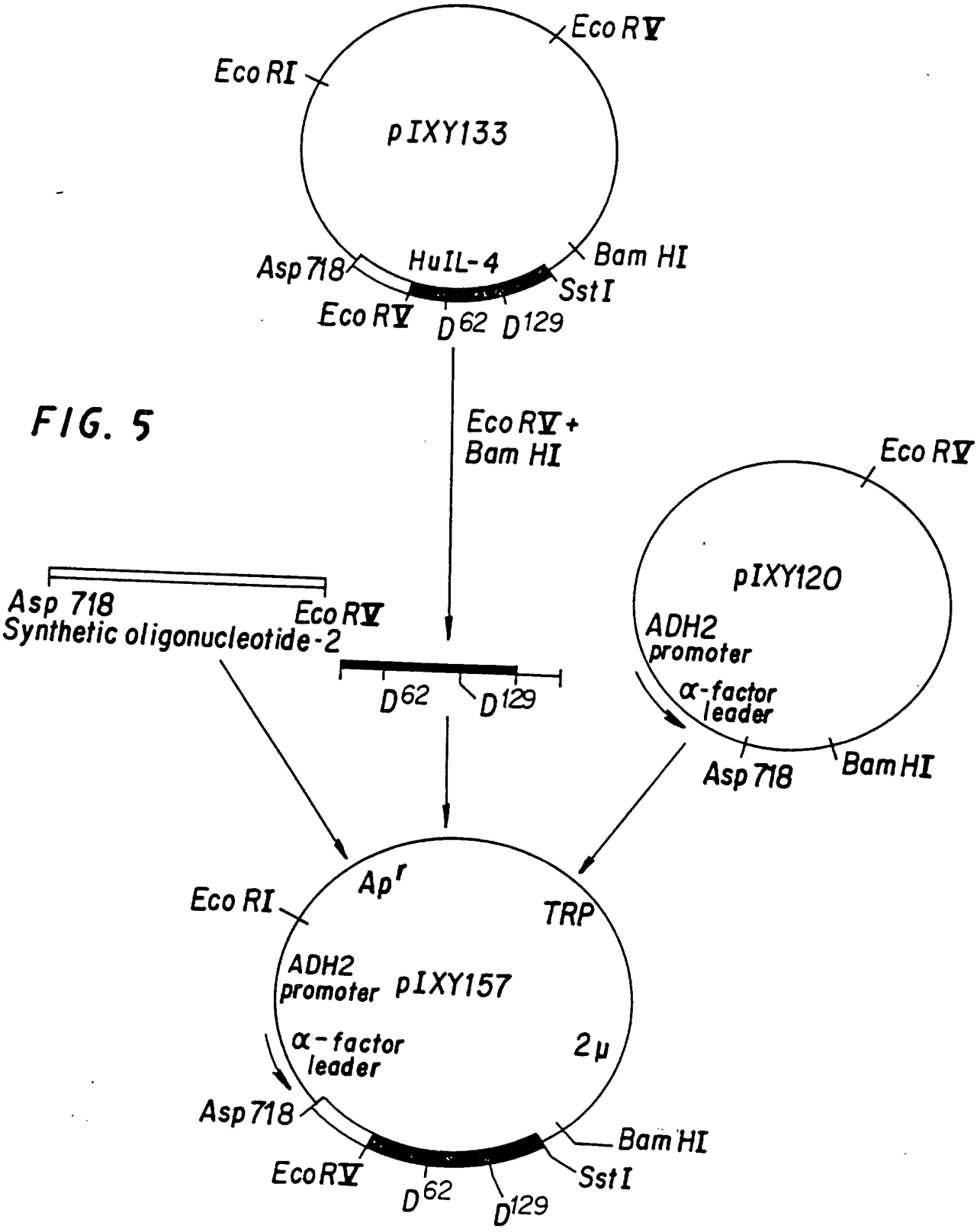
Eco RI
Sst I
D129
D62
Asp718
Eco RV

FIG. 4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pIXY133
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HuIL-4
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Sst I
Eco RV
D 62
D 129
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Bam HI
Eco RV
pIXY120
ADH2
promoter
α-factor
leader
Asp 718
Bam HI
Asp 718
Synthetic oligonucleotide-2
Eco RV
D 62
D 129
Ap r
Eco RI
TRP
ADH2
promoter
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Eco RV
Sst I
D 62
D 129

FIG. 5

FIG. 6

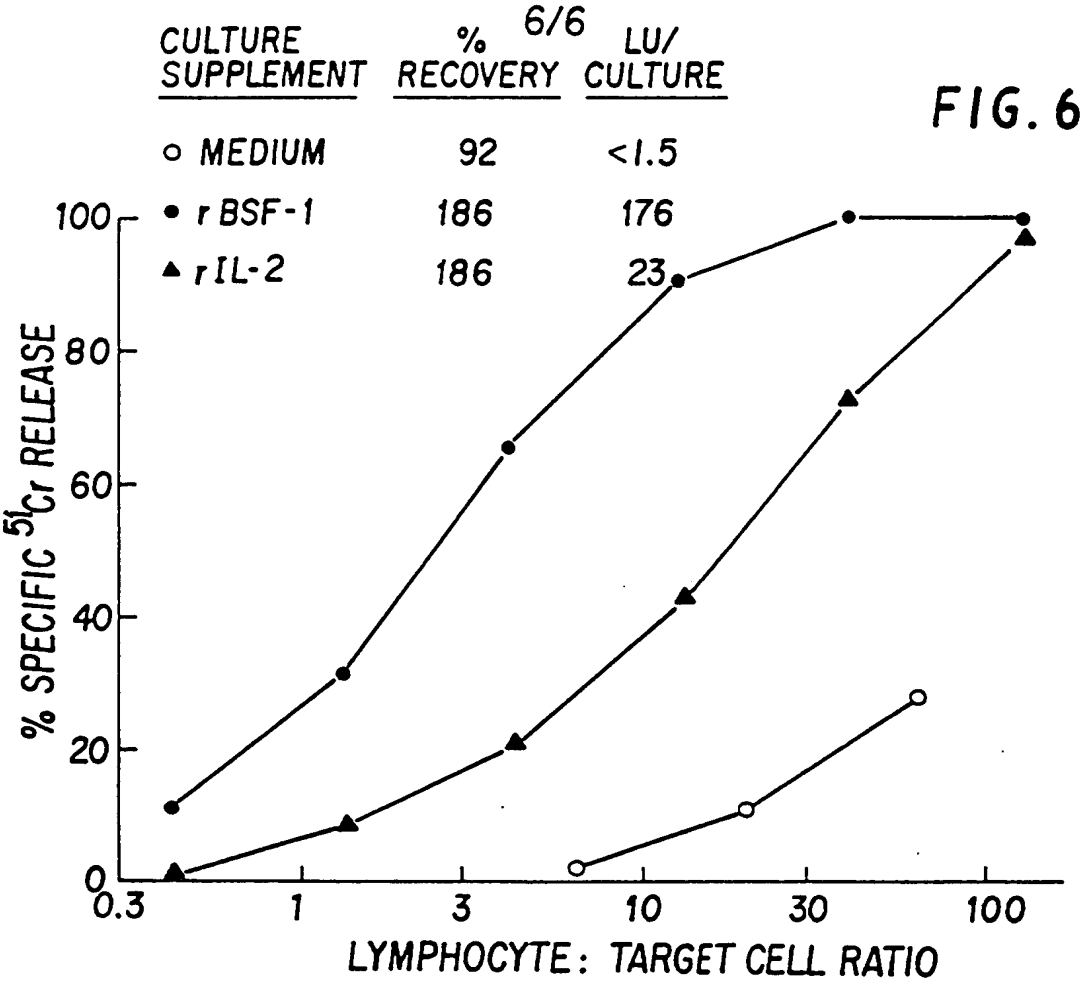
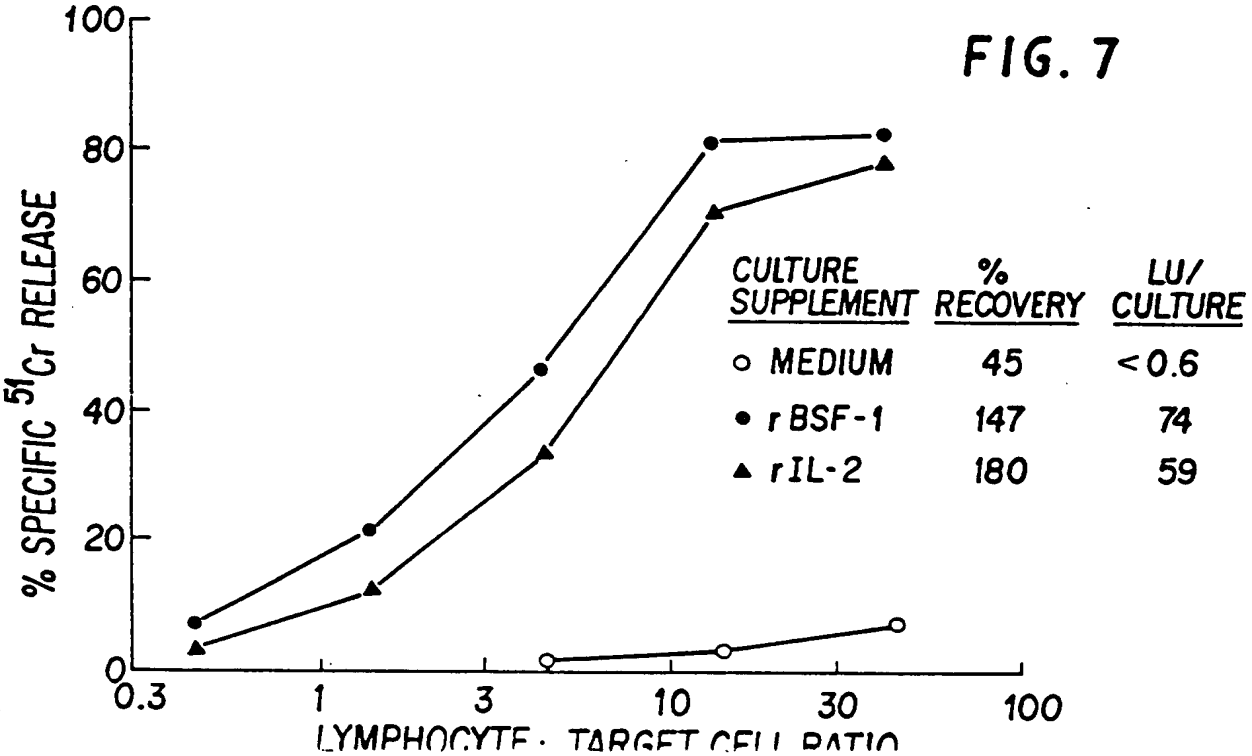

CULTURE SUPPLEMENT
% RECOVERY
LU/ CULTURE
○ MEDIUM 92 <1.5
● r BSF-1 186 176
▲ r IL-2 186 23
% SPECIFIC 51Cr RELEASE
100
80
60
40
20
0
0.3
1
3
10
30
100
LYMPHOCYTE: TARGET CELL RATIO

FIG. 7


% SPECIFIC 51Cr RELEASE
100
80
60
40
20
0
0.3
1
3
10
30
100
LYMPHOCYTE: TARGET CELL RATIO
CULTURE SUPPLEMENT
% RECOVERY
LU/ CULTURE
○ MEDIUM 45 <0.6
● r BSF-1 147 74
▲ r IL-2 180 59

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/03114

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07K 13/00;C12P 21/00,21/02;C12N 15/00,1/00;A61K 37/00
US CL : 530/351;435/68,701,172.3,320;514/12

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	530/351; 435/68,70,172.3,320; 514/12

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

COMPUTER SEARCH, CAS, BIOSIS APS: INTERLEUKIN-4-MUTEINS, GYCOSYLATION

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	Nature, Vol. 319, issued 20 Feb. 1986 (London, England), (NOMA ET AL), "Cloning of cDNA encoding the murine IgG1 induction factor by a novel strategy using SP6 promoter", pages 640-646.	1-16
Y	Proc. Natl. Acad. Sci. USA, Vol. 83, issued April 1986, (Washington, D.C.), (LEE ET AL), "Isolation and Characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor activities and T-cell- and mast-cell-stimulating activites," pages 2061-2065.	1-16
Y	Pro. Natl. Acad. Sci. USA, Vol. 83, issued August 1986, (Washington, D.C.), (YOKOTA ET AL), "Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that expresses B-cell-and T-cell stimulating activities," pages 5894-5898.	1-16

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
21 January 1988	17 MAR 1988
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Alvin E. Tanenholtz

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	US, A, 4,518,584 (MARK ET AL), 21 May 1985, see particularly Col. 20, lines 19-60.	1-16
Y	The EMBO Journal, Vol. 5, issued June 1986, (Oxford, England), (MIYAJIMA ET AL), Expression of murine and human granulocyte-macrophage colony stimulating factors in S. cerevisiae: mutagenesis of the potential glycosylation sites," pages 1193-1197.	1-16

- * -

LEMENTARY
C07K15/00F24F14 **EAN SEARCH REPORT**

Application number
EP 88 90 0410

Office

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
P,X	EP-A-0 230 107 (SCHERING BIOTECH CORP.) * Claims, in part. 2 * --	1,2, 4-7, 13,14	C 07 K 13/00 C 12 P 21/00 C 12 P 21/02 C 12 N 15/00 C 12 N 1/00 A 61 K 37/00
A	PROC. NATL. ACAD. SCI. USA, vol. 81, August 1984, pages 4642-4646 A.J. BRAKE et al.: "Alpha-factor-directed synthesis and secretion of mature foreign proteins in Saccharomyces cerevisiae" * Results page 4643 * ------- ------	3	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 12 N

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely:
Claims searched incompletely: 8-12, searched only for the in vitro applications
Claims not searched:
Reason for the limitation of the search:

Method for treatment of the human or animal body by surgery or therapy (See art. 52(4) of the European Patent Convention)

Place of search	Date of completion of the search	Examiner
The Hague	19-06-1990	CHAMBONNET

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO Form 1505.1 03.82

European Patent Office

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.

☐ Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claims:

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims 1-6, and 7,13-15 partially: Analogs of hIl_4 and pharmaceutical compositions containing analogs of hIl_4.

2. Claims 7,13-15 partially: Pharmaceutical compositions containing Il_4

☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

☒ None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims: 1-6 and 7, 13-15 partially

CLAIMS

What is claimed is:

1. A human interleukin-4 (hIL-4) analog protein capable of inducing proliferation of and lytic activity in a population of antitumor cytolytic T lymphocytes, having a mutant amino acid sequence which is substantially homologous to the wild-type amino acid sequence of hIL-4, wherein at least one occurrence Asn-A^1-Z in the wild-type sequence has been replaced in the mutant sequence by Asn-A^2-Y or X-A^2-A^3, where

A^1, A^2, and A^3 are the same or different and can be any amino acid,

X is any amino acid not Asn;

Y is any amino acid not Z; and

Z is Ser or Thr.

2. An hIL-4 analog protein according to Claim 1, hIL-4(Asp^{62}, Asp^{129}).

3. An hIL-4 analog protein according to Claim 2, GluAlaGluAla-hIL-4(Asp^{62}, Asp^{129}).

4. A DNA sequence encoding an hIL-4 analog protein according to any of Claims 1-3.

5. A recombinant expression vector comprising a DNA sequence according to Claim 4.

6. A process for preparing a hIL-4 analog protein, comprising culturing a microorganism transformed with a recombinant expression vector according to Claim 5 under conditions promoting expression.

7. A pharmaceutical composition for inducing proliferation of and lytic activity in a population of antitumor cytolytic T lymphocytes, comprising a biologically effective quantity of IL-4 or an analog hIL-4 according to any of Claims 1-3 in combination with a physiologically acceptable carrier or diluent.

8. A method for inducing proliferation of and lytic activity in a population of cytolytic T lymphocytes, comprising contacting T cells with a composition comprising a biologically effective quantity of IL-4 or an analog hIL-4 according to any of Claims 1-3 in combination with a physiologically acceptable carrier or diluent.

9. A method according to Claim 8, wherein the lymphocytes are previously activated by exposure to virus-associated antigen.

10. A method according to Claim 8, wherein the lymphocytes are previously activated by exposure to tumor antigen.

11. A method according to Claim 10, wherein the lymphocytes are induced and expanded ex vivo and readministered to a patient in adoptive immunotherapy.

12. A method for inducing proliferation and activation of antitumor or antiviral cytolytic T lymphocytes in a mammal, comprising administering a therapeutically effective amount of IL-4 or an analog hIL-4 according to any of Claims 1-3.

13. An antiviral composition comprising a biologically effective amount of IL-4 or an analog hIL-4 according to any of Claims 1-3 and a physiologically acceptable carrier or diluent.

14. An antitumor composition comprising a biologically effective amount of IL-4 or an analog hIL-4 according to any of Claims 1-3 and a physiologically acceptable carrier or diluent.

15. An antitumor composition according to Claim 14, additionally comprising a therapeutically effective quantity of T lymphocytes expanded ex vivo in the presence of IL-4 or IL-2.